ENERGY SOURCES OF AMPHIBIAN EMBRYOGENESIS

by

SØREN LØVTRUP[1]

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1. INTRODUCTION

The morphogenetic processes underlying the transformation of the fertilized egg into the self-sustaining young animal have, because of their fundamental importance among biological processes, been the subject of numerous investigations. Much work has also been devoted to discovering the energy sources[1] which drive embryogenesis, and this problem being in principle a matter of rather simple chemical analyses, one might presume that it had been settled long ago. The following discussion will show this to be not entirely true, but it is hoped that the data obtained in this work may assist in deciding between the discordant views. A great amount of experimental data on this subject has been compiled and reviewed by NEEDHAM in his two books on chemical embryology (32; 33). NEEDHAM here advocates the generalization that in embryonic development the succession of energy sources is: carbohydrate, protein, fat. However, several authors have reported that total nitrogen (*TN*) remains remarkably constant during embryonic development in several amphibian species, in obvious disagreement with a generalization including protein as an energy source. It was therefore considered desirable to reinvestigate the

[1] It is common usage in biological literature to use the expression 'energy sources' about the material which through combustion or otherwise is utilized by an organism to supply energy for all kinds of vital activities. This usage is quite unambiguous, but often, especially when the energy sources are stored in the organism, other expressions (e.g. reserve material) are used to denote the same concept. In the present work 'reserve material' will be used to designate the total amount of stored inactive material present in an object. The reserve material may be said to consist of two fractions, viz. the energy sources and the 'synthetic reserves', i.e. those substances which through assimilation are incorporated into the living protoplasm. Presumably these two fractions have almost the same qualitative composition, since many substances are known to be used in both ways.

The amounts of reserve material and synthetic reserves cannot be measured directly, but various indirect approaches have been employed to estimate the conversion of the synthetic reserves into active material.

The utilization of reserve material as energy sources has most often been measured by the changes occurring in the total mass of the object or in the content of some specified substance. These changes may not bear much relation to the actual energy consumption of the object; under certain circumstances considerable quantities of reserve material may be lost without any corresponding oxygen uptake (28), yielding no energy, or at least much less than might be expected from the observed changes.

question of energy sources during amphibian embryogenesis, using eggs and embryos of *Amblystoma mexicanum*.

Lest no misunderstanding arise, two points should be stressed at the outset. First, constancy of *TN* will for most analytical methods mean that changes in *TN* remain smaller than 1–2 per cent, at least in cases where the samples are large enough to exclude biological variation. Constancy of *TN* does thus not entirely exclude the possibility that protein is combusted; it only allows the conclusion that the amount utilized is negligible compared with the total amount present. In that case, however, it seems unwarranted to introduce protein among the energy sources. Secondly, whereas constancy of *TN* may reasonably lead to the conclusion that protein is not an energy source, it does not, of course, imply that no protein metabolism occurs. All experimental evidence suggests on the contrary that a variety of proteins and other *N*-containing compounds are synthesized during all phases of development. A certain amount of nitrogenous material may, as a result of these processes, be lost to the medium where it may be easily detectable even if it constitutes a negligible amount of *TN*. As mentioned, it seems misleading to speak of protein as an energy source under these circumstances.

Some of the earlier results are being used for comparison with and for interpretation of the data obtained in the present work; it will therefore be useful to give a brief summary of the more pertinent results.

2. PREVIOUS WORK

2.1. Utilization of carbohydrate, fat, and protein.

The present review of the literature on the energy sources during amphibian embryogenesis is not complete. It has been intended as far as possible to select the results which give a coherent picture of the energy metabolism, although of course the diverging results on protein utilization will be presented. A more detailed discussion may be found in NEEDHAM'S books (32; 33).

The investigations of BRACHET and NEEDHAM (12) on *Rana fusca*, and of GREGG (15) on *Rana pipiens*, agree in showing that no glycogen disappears before gastrulation, while between one third and half of the glycogen present at fertilization has disappeared at the time of hatching. GREGG's results furthermore show that the loss in total reducing carbohydrate is nearly identical with the loss in glycogen, the latter thus apparently being the energy source. Glycogen was found to account for about two-thirds of the total carbohydrate (*TC*) in the egg.

In none of these investigations was the analysis continued to the end of the pre-feeding development, but some older results (FAURÉ-FREMIET and DRAGOIU (13)) have shown that in *Rana temporaria* at this time only traces of glycogen are present. Combining this result with the finding of GREGG (15) that only glycogen is consumed we may sketch the carbohydrate utilization as occurring in the following way (*Rana*): During segmentation, no consumption; one-third of *TC*, or half of the glycogen, is utilized during the embryonic stages (gastrulation to hatching), and the other half is combusted during the remaining part of the pre-feeding (larval) development. After this about one-third of *TC* should remain.

Relatively few determinations have been made of the utilization of fat during amphibian embryogenesis. There is, however, essential agreement between BIAŁASZEWICZ and MINCÓVNA (7), who found no loss in fatty acids before hatching, whereas 50-60 per cent disappeared during the later stages (*Rana fusca* and *Rana temporaria*), and BARTHÉLEMY and BONNET (5), who found the total loss to range from 25 per cent to 55 per cent (*Rana fusca*). PARNAS and KRASINSKa (34) confirmed the results of BIAŁASZEWICZ and MINCÓVNA by showing that no loss of fat occurred during the pre-hatching period, while FAURÉ-FREMIET and DRAGOIU (13) found a decrease of about 25 per cent. ATLAS (1) has calculated from changes in dry weight and the corresponding total oxygen consumption that very little fat is consumed before hatching (*Rana pipiens*). The results concerning the utilization of protein disagree more than the results concerning carbohydrate and fat consumption. BIAŁASZEWICZ and MINCÓVNA (7) found that at the time of hatching 9 per cent *TN* had disappeared. Calculated as protein this amount corresponded to the loss in dry weight, which led them to the conclusion that protein is the only energy source during this first part of the development. FAURÉ-FREMIET and DRAGOIU (13) calculated that 12 per cent of the yolk (protein) had been consumed at the time of hatching. Against these results stand those of GORTNER (14) on Cryptobranchus allegheniensis, and of GREGG and BALLENTINE (16) on *Rana pipiens* which showed only a very slight, if any, change in TN from fertilization to hatching. The same was found by WILLS (41) who, working with *Tritorus torosus*, *Amblystoma maculatum*, and *Rana pipiens* determined the average *TN* of embryos and larvae from fertilization to metamorphosis. WILLS found no significant changes in TN before hatching.

BRACHET (11) has recorded a very slight accumulation of nitrogen-containing substances in the surrounding medium during the first part (up to the neurula stage) of the development of *Rana fusca*. This loss is small enough to agree with an apparent constancy of *TN*, and may, as mentioned, be regarded as indication of the protein metabolism going on.

At the end of the pre-feeding larval development BIAŁASZEWICZ and MINCOVNA recorded a loss of about 50 per cent *TN*, while BAR- THÉLEMY and BONNET found a loss of 25 per cent *TN* at the time when the external gills disappear (*Rana fusca*). Contrary to the authors who found losses in *TN*, WILLS supplied food to the larvae, *i.e.* it may be assumed that he has eliminated possible losses in *TN* due to starvation. WILLS did not find any decrease of *TN* in *Amblystoma maculatum*. The *TN* determinations on *Triturus torosus*, *Amblystoma tigrinum* and *Rana pipiens* are rather varying, and it is therefore difficult to get an accurate estimate of the changes in *TN*. A comparison of the average values of *TN* before hatching and from this time until *TN* begins to increase indicates losses in these three species ranging from 6 to 17 per cent of *TN*. WILL'S results therefore suggest that the losses described above are partly due to starvation.

It is seen that the claim for constancy of *TN* during the embryonic (pre-hatching) development is much better supported than the claim for decrease. There is therefore hardly any doubt that no-or very little-protein is combusted before hatching. With respect to protein consumption during larval development, the question is to some extent undecided by the available results.

It seems possible from the literature cited above to outline the utilization of energy sources during amphibian embryogenesis in the following way: During the segmentation stages neither carbohydrate, fat, nor protein is consumed. The respiratory metabolism during this phase is a special type, giving rise to very low R.Q. values (BARTH (2)). Simultaneously with the onset of gastrulation carbohydrate concumption starts, and it continues, first at an increasing, later at a decreasing rate, due to the depletion of the glycogen stores. As the rate of metabolism continues to increase –reflected by the increasing rate of oxygen uptake– the embryos start to use their fat reserves as an energy source. The fat consumption starts shortly before the time of hatching. Views are divided concerning the extent and the

time of protein consumption, but it seems certain that little nitrogen is lost before hatching.

It is seen that these results, if they can be upheld, invalidate the succession of energy sources suggested by Needham.

2.2. Utilization of yolk.

It is common to read in the literature that the increasing respiratory activity and other visible or measurable changes occurring during embryogenesis reflect the transformation of "inert yolk" to "active protoplasm". Presumably such statements imply that "yolk" means any kind of reserve material present in the fertilized egg. If by "yolk" we understand a chemical substance (or maybe several substances) characterized by definite physico-chemical properties, it will become less ambiguous, and also more difficult to advance such statements about yolk utilization. Yolk in a more narrow sense is known to be phospho-lipo-protein, insoluble in dilute salt solutions, and localized in the yolk-platelets, which are gradually broken down during development. Yolk does not contain reducing carbohydrate (GREGG and LØVTRUP (17)), and so long as metabolism is sustained by carbohydrate consumption, there is thus no reason to assume *a priori* that yolk is consumed to supply energy.

GREGG and BALLENTINE (16) attempted to determine the yolk utilization by measurements of non-extractable nitrogen from fertilization to hatching, and they found this quantity to remain constant during this period. The investigation was not, and could not, be extended to the pre-teeding larval development, because the formation of various insoluble proteins would obscure the results.

It is generally assumed that yolk-fat may be used for energy supply, and it might be argued that fat combustion would furnish a proof of yolk utilization. A simple calculation will show, however, that this is far from being the case. From the results of GREGG and BALLENTINE (16) and GREGG and LØVTRUP (17) it is seen that the yolk nitrogen (*NEN*) constitutes about 70 per cent of *TN*. The ratio fatty acid/total nitrogen in whole eggs is from 1 to 1.4 times higher than in isolated yolk (17). This implies that 30 to 50 per cent of the fatty acids is present outside the yolk. These values are in the same range as found for fat consumption (see the preceding section). Thus, as far as carbohydrate and fat is concerned the total amounts consumed during at least the major part of the development may be found in the cytoplasm.

Phosphoprotein is not normally present in animal tissues. It should therefore be possible to determine at least one aspect of yolk utilization by measurements of phosphoprotein phosphorus (*PPP*), as was done by KUTSKY (24) on *Rana pipiens*. KUTSKY's measurements did not extend beyond the pre-hatching period, but Barth and BARTH (3) who devised a very elegant method to determine phosphoprotein enzymatically, have measured *PPP* during the entire pre-feeding development (*Rana pipiens*). KUTSKY's results do not indicate any great changes during the embryonic phase of development, while BARTH and BARTH found that the utilization of phosphoprotein occurs during larval development.

The aim of the present paper has been to get some information about the amount and the nature of those substances, which through combustion supply energy to the developing embryos. The values given above indicate that a sufficient quantity of fat (and the same holds for protein) is found outside the yolk to supply all the material consumed until the end of the present experiments (cf. section 6.1). It is therefore impossible to conclude anything about yolk utilization from the observed losses in carbohydrate, fat, and protein.

There are, however, two questions which might be elucidated by measuring the changes in *PPP*. The first one is whether the eventual protein combustion is related directly to the yolk utilization as reflected by *PPP* measurements. The second question is based on the observation by BARTH and BARTH (3; 4) that yolk contains energy-rich phosphate bonds, thus being a potential energy source. It seemed interesting by some approximate calculations to compare the relative contribution to the total energy consumption which might be supplied by this source. For both these purposes determinations of *PPP* were needed, and it was therefore decided to make these analyses also in the present work.

3. THE BASIS OF THE PRESENT EXPERIMENTAL ATTACK

The literature does not seem to contain any attempts to compare the oxygen consumption with the actually measured disappearance of carbohydrate, fat, and possibly protein. Such a comparison might, besides deciding the question about the succession of energy sources, yield information about the extent to which the lost substances have been oxidized. Both these questions are very interesting, and an attack similar to that outlined above has therefore been attempted in the present investigation.

Direct chemical analyses throughout the development would, however, require a considerable amount of biological material if reasonable average values were to be obtained. Since such large quantities of eggs were not available, it was chosen to estimate the consumption of energy sources indirectly, by means of determinations of reduced weight (RW) on the Cartesian diver balance (ZEUTHEN (42)). The reduced weight of an object is defined as the weight, W, of the object minus the weight of the volume of medium (water) it displaces, $v \bullet \varphi_M$ where v is the volume of the object and φ_M the density of the medium (LINDERSTRØM-LANG and HOLTER (25)). Thus

$$RW = W - v \bullet \varphi_M = v(\varphi_0 - \varphi_M) = W\left(1 - \frac{\varphi_M}{\varphi_0}\right),$$

where φ_0 is the density of the object.

In the present case the changes in RW (μg per embryo) and in the rate of oxygen consumption (Q^*O_2, dimension: μl O_2, (0°, 760 mm)/hour per embryo) have been followed simultaneously (one determination of each quantity every day) on a relatively large number of animals through their entire pre-feeding development. By using many embryos, and moreover, essentially the same individuals from day to day, the effect of individual variation may reasonably be expected to have been excluded, and the values obtained can thus be regarded as fair average values.

Some embryos died or developed abnormally during the experiment, and were replaced by other embryos. Less than half of the original sample of 50 animals were left at the end of the experiment. Mostly one or two animals were replaced at a time, and such small number cannot influence the average of 50 measurably, but at the end of the development, when the total number of replacements was relatively great, a certain, slight error may have been introduced.

To illustrate how these results could be used for calculation of the consumption of energy sources it is necessary briefly to discuss some of the implications and assumptions inherent in the interpretation of RW data.

3.1. The use of RW measurements.

Since the introduction of RW measurements in work on biological objects, first by means of the density gradient (LINDERSTRØM-LANG and LANZ (26)), and later by means of the Cartesian diver balance (42), the usefulness of determinations

of this quantity (*RW*) for various purposes has been demonstrated (21, 20, 31).

Generally, in all cases where metabolism is being analysed by following the corresponding weight changes, *RW* measurements offer several advantages. A comparison with the two other possible weight quantities, dry weight (*DW*) and absolute or "wet" weight (*W*) will directly show its superiority. Admittedly *DW* is as well defined as *RW*, but *DW* measurements require that the object be killed, thus making great demands on the amount of material. Determinations of *W* can be made repeatedly on the same object, but this quantity is not well defined for aquatic animals, and is furthermore a function of the water content, thus complicating the interpretation of the results.

Consumption of carbohydrates and proteins, which have densities higher than 1, will cause a fall in *RW*, whereas disappearance of neutral fat, having a density below 1, will be reflected as an increase in *RW*. This feature makes *RW* determinations especially fit for following changes due to metabolic processes, as shown by HOLTER and ZEUTHEN (21), but at the same time, it increases the demands as to the accuracy of the method.

3.2. Calculations.

The *RW* of an object of known composition can be calculated from

$$RW = \Sigma_i W_i \left(1 - \frac{1}{\varphi_i}\right) \qquad \textbf{(1)}$$

a procedure introduced by BOTTELIER, HOLTER, and LINDERSTRØM-LANG (9), and in which W_i, and φ_i, denote weight and density, respectively, of a component i. Disregarding all components but proteins (= N-containing substances): P; carbohydrate: C; fats[2]: F; and salts (ash): S –water is not included in *RW*– and inserting the values φ_P: 1.35, φ_C: 1.5, φ_F: 0.8 and φ_S: 2.0, formula (1) will read:

$$RW = 0.26\, W_P + 0.33\, W_C - 0.25\, W_F + 0.5\, W_S \qquad \textbf{(2)}$$

This expression was used for the analysis of the results obtained by *TN* and *RW* determinations on barley roots (9), and similarly

[2] Only neutral fats contribute to *RW*; phosphatides, cholesterol etc. have densities only slightly different from 1, and can therefore be neglected except in cases where they occur in large quantities.

by HOLTER and ZEUTHEN (21), who determined *RW* and oxygen consumption on starving amoebae. In both cases, however, the number of variables was too great to allow direct calculation of changes in the composition of the object, even when W_S was considered constant. The same will be true in the present case, if all three components are combusted simultaneously. Fortunately, as was mentioned in section 2.1, it seems that the more convincing results regarding this question tend to agree in showing that *TN* remains constant for at least a considerable part of the development. It was beyond the scope of the present paper to make a special investigation of this problem, but since it is fundamental to the analysis of the present data, it was considered desirable to make a few KJELDAHL-determinations in order to confirm this point (see section 5.2). Taken together with the results mentioned above these analyses support the claim that *TN* remains constant during the major part of the development, although it is seen that the value may eventually decrease. W_S is generally considered constant in *RW* calculations (cf. section 3.3), and if W_P may also be considered constant, we can write the following expression for the change in reduced weight (ΔRW):

$$-\Delta RW = 0.33\,\Delta W_C - 0.25\,\Delta W_F; \qquad \textbf{(3)}$$

ΔW_C and ΔW_F are the quantities of carbohydrate and fat consumed. If the oxidation of carbohydrate and fat is complete, we know that 1 µg glucose will use 0.83 µl and 1 µg fat 2.02 µl O_2 (0°, 760 mm). In that case we have the following relationship between total oxygen consumption (ΔO_2) and carbohydrate and fat combustion:

$$\Delta O_2 = 0.83\,\Delta W_C + 2.02\,\Delta W_F \qquad \textbf{(4)}$$

Equations (3) and (4) were used to calculate ΔW_C and ΔW_F, during embryonic development. It was then found that the rate of carbohydrate consumption, after having reached a maximum, rapidly approached zero, while correspondingly the rate of fat consumption increased (cf. fig. 5).

The calculations then showed that for a short time afterwards the respiratory metabolism was pure fat oxidation, but rather soon the rise in *RW* corresponding to fat utilization changed into a decrease. This could only be explained as due to combustion of proteins since all the combustible carbohydrate (glycogen) had disappeared at this time (cf. section 5.3). The calculations were therefore continued

on the assumption that now only fat and protein was combusted. For this purpose two equations similar to (3) and (4) were used:

$$-\Delta RW = 0.26\,\Delta W_P - 0.25\,\Delta W_F \quad \textbf{(5)}$$

$$\Delta O_2 = 0.95\,\Delta W_P + 2.02\,\Delta W_F \quad \textbf{(6)}$$

In using equations **(3)** – **(6)** for calculations of the consumption of energy sources a number of assumptions besides those already mentioned have to be made. In the following these assumptions will be discussed in order to see to what degree they can be expected to hold in the actual case.

3.3. A critical analysis of the method.

The basic assumptions of the method are partly associated with the use of RW, and partly with the use of the oxygen consumption, as functions of the consumption of carbohydrate, fat, and protein.

Apart from the number of compounds which were neglected in the expression for RW, it was furthermore assumed that the amount of salts remains constant. This may not be absolutely true, since *e.g.* KROGH, SCHMIDT-NIELSEN and ZEUTHEN (22) have shown a loss of osmotically active substance to occur during the development of *Rana* embryos. The loss was accounted for by loss of Cl^-. The maximum value of the Cl^- loss was reached at the beginning of the gastrulation, after which the amount of Cl^- per embryo increased again. The maximum loss corresponded to about 25 per cent of the total osmotic concentration. If we assume that half of the "ash" (cf. section 5.1) is osmotically active, the maximum decrease of RW in the present object should be about 6 μg, which may be neglected without committing any serious error.

Another assumption is that no gas bubbles are formed in the object. Generally this is probably true for amphibian embryos and larvae, but the author has observed, in a few cases, some larvae in the very last part of their development which floated on the surface of the water. This was no doubt due to gas bubbles inside the tadpoles. The explanation of this finding is either that the tadpoles had swallowed air, or that gases were formed due to intestinal infection. Such larvae could not, of course, be weighed on the diver balance, but it cannot be excluded that similar gas formation, but to a smaller extent, has occurred in other larvae. When the sample of eggs was weighed in two or more groups, such gas formation would expectedly be

reflected in greatly diverging results of simultaneous weighings. This was actually found during the last few days of the present experiment, but these values are not included in the calculations, and neither are they shown in fig. 4.

The equations used for ΔRW ((3) and (5)) do not account for the possibility that processes may occur which give rise to changes in the density of the substances concerned, e.g. hydration processes. Whereas the *RW* changes at 18 °C used in the present calculations can be accounted for entirely by metabolic processes, a set of curves has been obtained, which showed a very definite decrease unaccountable for by oxidative metabolism (28). In a third batch of eggs, which was used for peptidase determinations, no unaccountable changes were observed (29).

The main assumption involved in the use of the oxygen uptake data is that the oxidation is complete. This assumption is very often made in experiments on respiratory metabolism, *e.g.* in interpretation of *R.Q.* values, but the present experiments, and even more so those in the following paper (28) show, in agreement with previous work, that incomplete oxidations are normally occurring. In view of this it is necessary to see what results our calculations will give when incomplete oxidations occur.

If we assume $\Delta W_C = 30\mu g$, and $\Delta W_F = 0\mu g$, then we shall find $\Delta RW = -10\mu g$. The expected oxygen consumption should be $30 \times 0.83 = 25\mu l$. If the carbohydrate is only 50 per cent oxidized we shall find $\Delta O_2 = 12.5\mu l$; for $\Delta RW = -10\mu g$, we then find from equations **(3)** and **(4)**: $\Delta W_C = 26.6\mu g$, and $\Delta W_F = -4.7\mu g$. If $\Delta W_C = 30\mu g$, and $\Delta W_F = 30\mu g$, then $\Delta RW = -2.5\mu g$. If carbohydrate is 50 per cent, and fat completely oxidized, then the O_2-consumption will be $\Delta O_2 = 12.5 + 30 \times 2.02 = 73.1\mu l$. In that case the equations will give $\Delta W_C = 26.6\mu g$ and $\Delta W_F = 25.3\mu g$. If $\Delta W_C = 0\mu g$, and $\Delta W_F = 30\mu g$, the ΔRW will be $30 \times 0.25 = 7.5\mu g$. The oxygen consumption will, for 50 per cent oxidation be $30 \times 2.02 \times 0.5 = 30.3\mu l$. Inserting these values in our equations will give $\Delta W_C = -8.6\mu g$ and $\Delta W_F = 18.5\mu g$.

It must be emphasized that these calculations are based on the assumption that the incompletely oxidized intermediates are lost from the embryos; if they were accumulated they would presumably be oxidized sooner or later, in which case no errors would be involved in the use of the present method.

The above calculations show that incomplete oxidations will give rise to values of consumption of carbohydrate and fat which are too low; even values indicating an increment rather than utilization may be obtained. This fact must be borne in mind when the data are interpreted. When we face the possibility that unaccountable changes in *RW* and incomplete oxidations may occur, it becomes

clear that the results of the calculations must be checked carefully before any conclusions are drawn. One way of controlling the results is to make some chemical determinations to see if the calculated results and the direct ones are in the same range. For this purpose some carbohydrate analyses have been made. Another way of checking the results may be obtained by comparison with results found by other authors. Both of these approaches will be used here.

One last factor needs to be stressed, i.e. that the relative changes in RW to be expected are small. A rough estimation using the data given here indicates that of the material present in the fertilized egg about two-thirds (dry weight) is present in the resulting larva, while one-third is consumed to yield the energy necessary for the developmental processes.

4. MATERIAL AND METHODS

4.1. Biological material.

The present experiments have been performed on developing eggs , embryos , and larvae of the axolotl, *Amblystoma mexicanum*. Shortly after laying, the jelly was removed and the eggs transferred to ERLENMEYER flasks, each containing then 10-25 individuals in 25-50 ml tap water. The flasks were immersed in a water thermostat at 18 °C. In all other respects the care of adult animals and embryos was similar to that described by KRUGELIS (23) and by GREGG and LOVTRUP (17). The morphological staging of the embryos was done in accordance with the series for *A. punctatum* and *A. tigrinum* worked out by HARRISON and with the series for *A. punctatum* published in RUGH's book (35). The number of embryos in the samples used in the experiment was, as mentioned, about 50.

4.2. Determination of reduced weight (RW).

4.21. Giant diver balance technique.

When the diver balance (ZEUTHEN (42)) was to be used for weighing amphibian embryos, it was necessary to introduce some modifications in the technique. First of all it was of course necessary to increase the size of the diver balance (about 1000 times), whereas the shape of ZEUTHEN'S polystyrene-glass balance was conserved. It is easy to make glass parts of these dimensions, but it was found quite impossible to blow sufficiently big cups from polystyrene solutions.

It was found that the biggest glass bulbs on which ZEUTHEN's technique of blowing cups could be used, had a diameter of 3-4 mm, while the big balances used in the present work had a diameter of about 10 mm.

Two approaches were tried in establishing a new technique. The first was to melt some sugar, dip the bottom of a test tube in the sugar and let it cool so that a hard layer of sugar was formed on the outside. The test tube was then dipped many (~40) times in a solution of polystyrene in benzene, and after each dipping the benzene was allowed to evaporate. When the layer of polystyrene was sufficiently thick, a cut was made around the test tube about 1 m from the bottom, and sugar and polystyrene above the cut were removed. A stream of water was then allowed to dissolve the sugar and the cup was free. This method was abandoned because it was tedious, and because the cups obtained tended to become turbid after some time. It also proved very difficult, in our hands at least, to avoid formation of air bubbles in the polystyrene during the evaporation of the benzene. The second method consisted in making a "sandwich" of the following layers, going downwards (fig. 1): A brass plate with a hole corresponding to the outside diameter of the finished cup, a rubber ring, a plate of 1 mm Plexiglass, a rubber ring, and a brass plate onto which a bicycle inner tube valve had been soldered. The brass plates were screwed together with four screws (two shown in the figure) so that the space between the valve and the Plexiglass was air-tight. A bicycle pump was screwed onto the valve, and an infrared heating lamp placed 1-2 cm above the hole in the upper brass plate. After 2-3 minutes of heating, the lamp was removed and pumping started. After a few tests it was learned how much pumping was necessary to force the soft plastic through the hole, forming very beautiful, almost hemispherical cups. After cooling, the cup was cut off with a razor blade along the edge of the hole. The cup was fixed to the glass part by dipping the latter in a Plexiglass solution and joining the two parts. After drying the cup stuck firmly to the glass. If it loosened after a prolonged stay in water, it could be refixed in the same way.

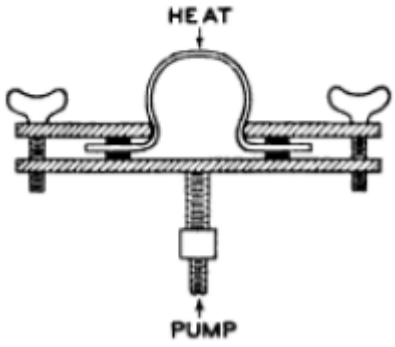

Fig. 1. Procedure for making Plexiglass cups for giant diver balances.

The balances used were designed to weigh 20-30 embryos simultaneously. The diver balance constant Z (LØVTRUP (27)) for one of the balances used was determined four times during the experimental period, and the values obtained, together with the dates on which they were measured are recorded in the following table. The standard weight was a piece of platinum wire (RW: 12.06 mg) calculated from weighings on an analytical microbalance.

Table 1.

Date.....	19.5	4.6	5.7	8.7
Z(mg)	345	346	346	347

It is seen from the table that the standard deviation is about 0.3 per cent, or at least 3 times better than can be obtained with the amoeba diver balance. There is a trend in the values of Z, which may be insignificant, but if not, accumulation of dust etc. is being recorded by the change in Z. The balance was neither cleaned nor refilled during the experimental period. The change in equilibrium pressure was about 10-15 cm BRODIE per month.

The floation vessels had of course to be larger to contain the giant balances, but effort was made to avoid unnecessary enlargement of the air space in the vessel. Thus the water level was maintained as high as possible, but visible, and the ground glass joint was made short, about 2 cm. These efforts were necessary to retain a reasonable working range for the manometer.

The manometer employed was of the type described by LØVTRUP and PIGON (31).

4.22. Weighing of amphibian embryos.

Very few modifications of the weighing technique described by ZEUTHEN (42) had to be introduced. One of these was that the braking pipettes (HOLTER (19)) used for handling the embryos, besides being bigger, were made with a "reservoir" (see fig. 2). This modification was introduced because it happened, when several embryos were transferred simultaneously in an ordinary pipette, that the first embryo reached the brake, causing most often destruction of the embryo and at the same time spoiling the brake. This difficulty was completely overcome by the "reservoir braking pipette".

Fig. 2. Reservoir braking pipette.

The transfer of the embryos required the simultaneous transfer of relatively large amounts of water. This necessitated that the water in which the embryos were transferred be preheated in the thermostat to avoid temperature changes of the water in the flotation medium. Special care had to be taken to keep the amount of flotation medium constant, since any changes in the liquid level in the vessels would cause an identical change in the measured equilibrium pressure. The procedure adopted to meet this requirement was to

take up water and embryos (generally 5-10) until the pipette was filled to the brake (which, being very wide in these big pipettes, cannot be clogged by water), to empty the pipette onto the balance, and to fill the pipette with water from the flotation vessel. In this way the amount of flotation medium was easily kept constant.

Freely swimming larvae cannot be weighed on the diver balance. It was therefore necessary to anesthetize the animals in the later stages of the development. This was done by exposing the larvae to a dilute solution of *MS 222* (tricaine methane sulfonate, Sandoz Chemical Works, Inc., New York) for 1-2 minutes before weighing. The larvae recovered quickly after careful washing in several changes of water. As mentioned above, the embryos of the sample were weighed in two lots. The values plotted in fig. 4 are the averages of two such determinations, which generally were in very close agreement.

*4.3. Determination of rate of oxygen consumption (**Q*O$_2$**).*

The ordinary WARBURG technique was used for determination of the respiratory activity. The exact amount of water in which the embryos were transferred to the vessels was determined by weighing. The respiration was measured at the same temperature as that at which the embryos were reared (18 °C). All the embryos of the sample were placed in the same vessel. The respiration was generally measured once every day, immediately after the embryos had been weighed. The readings were continued until a reasonable change (*i.e.* at least 2 cm BRODIE) in pressure was obtained. During the later part of the development this was obtained in one hour or less, but the first points on the respiration curve required longer stays in the WARBURG vessels.

The tadpoles and embryos stood the shaking very well, except during the earliest stages of the development, where the dejellified embryos proved very fragile. During these stages the shaking rate was kept at about 10-15 cycles per minute.

4.4. Determination of total nitrogen (TN).

The nitrogen determinations were performed by the ordinary PARNAS-WAGNER micro-KJELDAHL method on homogenates of jelly-free eggs, embryos, or larvae, ground in a small glass homogenizer. The homogenates were made of about 20 individuals; 3 aliquots,

each corresponding to about 5 embryos, were used for the KJELDAHL analysis. In some cases an aliquot of the rest was diluted and used for determination of total carbohydrate.

4.5. Determination of total carbohydrate (TC).

The orcinol reaction as described by SØRENSEN and HAUGAARD (37) was found useful for the present purpose after a slight modification in the range of the method. The procedure employed was the following: To a test tube was added 150 μl carbohydrate-containing sample, 300 μl 2 per cent orcinol in 20 per cent H_2SO_4 (*v*/*v*), and 2.5 ml 60 per cent H_2SO_4 (*v*/*v*). The tubes were heated for 30 minutes in a water bath at 80 °C. The extinction was read, directly or in some cases after addition of 2.5 ml 60 per cent H_2SO_4, in the BECKMAN spectrophotometer at 425 mμ. The maximum amount of carbohydrate, in glucose equivalents, was 15 μg. One determination required from one-half to one-fifteenth of an embryo, depending on the age of the embryo. The variations of duplicate determinations on the homogenates were greater than those on glucose solutions, suggesting the presence of disturbing factors in the homogenates. In order to obtain as accurate determinations as possible, duplicate determinations on three different concentrations of a homogenate (1:2:3) were made, yielding a straight line. The amount of carbohydrate was calculated from the slope of this line.

4.6. Determination of phosphoprotein phosphorus (PPP).

Phosphoprotein phosphorus was determined according to the procedure described by SCHMIDT and THANNHAUSER (36) with only few modifications. One of these was that the BERENBLUM and CHAIN (6) micro phosphate determination according to WIAME (40) was used. Using this method it was found unnecessary to wash the DELORY-precipitate of inorganic phosphate.

5. RESULTS

5.1. The composition of the eggs.

The measurements of *RW* and O_2-consumption were all carried out on one batch of eggs. The *TN* and *TC* determinations were partly carried out on eggs from this batch and partly on eggs and embryos from other batches. The variation in the results is rather great, but since the values from the various batches were all in the same range, they have been pooled in calculating the average content of *TN*:

198 ± 7µg. The *TC* content at the beginning of the development was found to be 202 ± 14µg "glucose". From these values we know that each egg contained 198 × 6.25 = 1240µg "protein" and 202 µg "glucose". In NEEDHAM'S book (32) one finds the following approximate composition of amphibian eggs: Water: 57 per cent, protein: 27 per cent, lipoids: 11 per cent (also BOYD (10)), carbohydrate: 4 per cent, and ash: 2 per cent. If we use these values we get, with the protein content as a basis: Lipoids: $1240 \times 11/27 = 500$µg, carbohydrate: $1240 \times 4/27 = 184$µg (actual value 202 µg) and ash: $1240 \times 2/27 = 92$µg. Of the lipoids only 70 per cent is neutral fat according to BOYD (10), the rest is phospholipids, cholesterol etc., which have densities near 1. The content of neutral fat should accordingly be about 350 µg. If we insert these values in formula (2) we should be able to calculate the approximate value of *RW* of the eggs. Doing this we obtain

$$RW = 1240 \times 0.26 + 202 \times 0.33 - 350 \times 0.25 + 92 \times 0.5 = 344\mu g$$

The value actually found for RW at the beginning of the experiment was 356 µg.

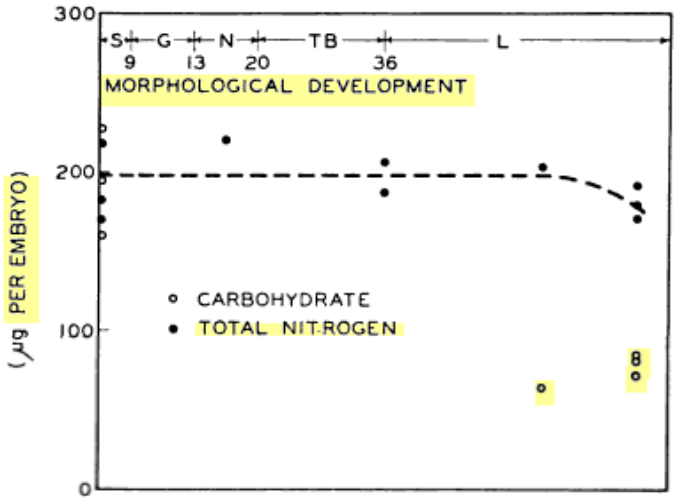

Fig. 3. Graphical representation of the nitrogen and carbohydrate analyses. *S*: Segmentation, *G*: gastrula, *N*: neurula, *TB*: tailbud, *L*: larva. The numbers refer to HARRISON stages. The time has not been used as abscissa, because embryos from different batches, having different rates of development, have been used. To enable plotting of these data in the same figure, the morphological development has been used as abscissa.

5.2. The changes in TN and TC found by chemical analysis.

The results obtained are shown in fig. 3. These results alone are not sufficiently extensive or accurate to permit definite conclusions, but together with the results of previous work, and with the results of the present calculations on the basis of *RW* and Q^*O_2-determinations they seem to indicate that *TN* remains constant for a considerable period during the development, whereas *TC* at the end of development is reaching a final value of about 30-40 per cent of the original.

5.3. Calculation of the metabolism from the Q^*O_2 *and RW determinations.*

The experimental results have been plotted in fig. 4. The oxygen consumption curve shows a smooth gradual increase except during neurulation and for some days shortly before the maximum. In drawing the curve the latter variations have been neglected, while the curve has been drawn horizontal during the period of neurulation. The reason for this is that this period of constant respiratory rate has been confirmed repeatedly by the author, and also by others (see the discussion, section 6.2).

The *RW* curve shows that the changes occurring are small, but the general

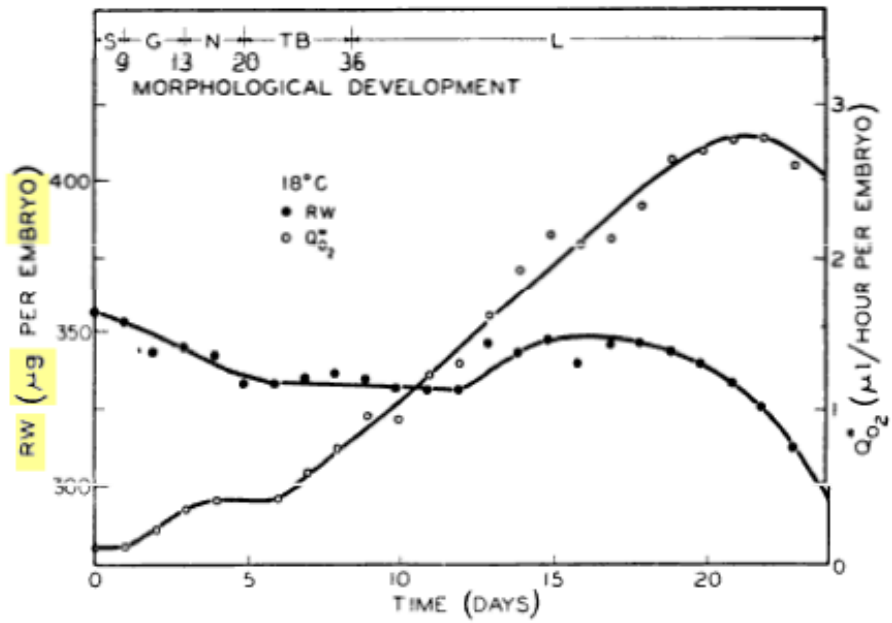

Fig. 4. Graphical representation of the RW and Q^*O_2 data. For explanation of abbreviations, see figure 3.

trend is a slight fall during the first part of development, followed by a temporary rise, which in turn is succeeded by a more steep decrease. This decrease starts about 3 days before the respiratory maximum.

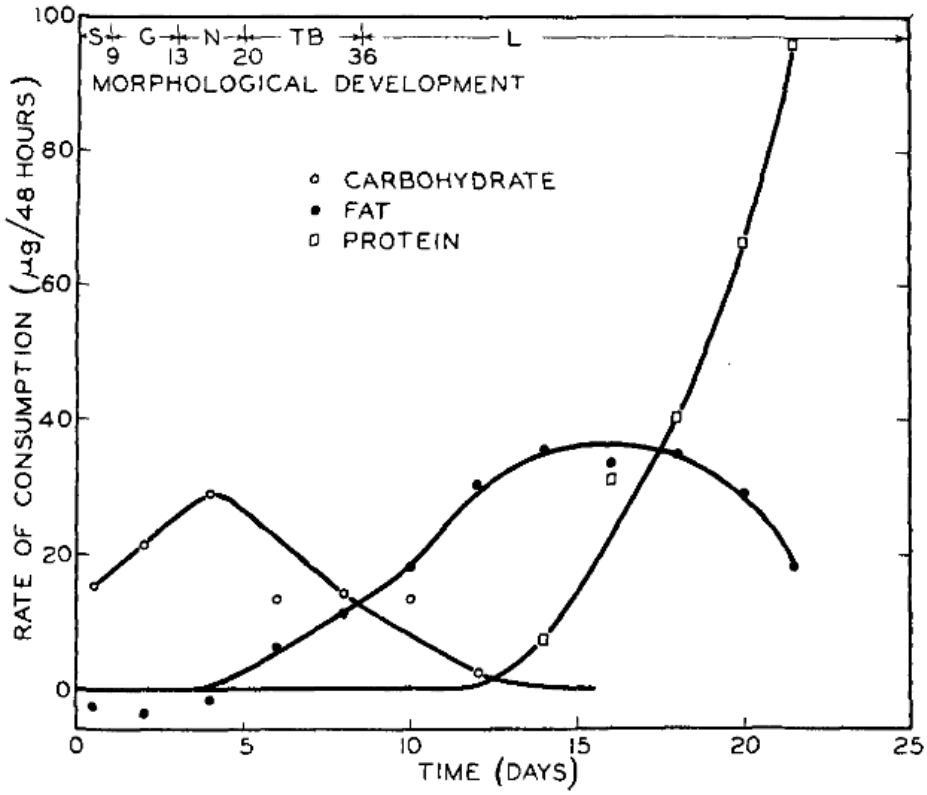

Fig. 5. Results of the calculations of the rates of consumption of energy sources. For explanation of abbreviations, see figure 3.

to equations (3)–(6). In these calculations the values inserted in the equations were read from the smooth curves in fig. 4. The values of $\Delta 0_2$ (oxygen consumption) were found by graphical integration of the Q^*O_2-curve. Values of of ΔW_C, ΔW_F, and ΔW_P were calculated for every second day. The results of these calculations are shown in figs. 5 and 6. In fig. 5 are plotted the amounts consumed during 48 hours. This figure shows that in the beginning the only energy source is carbohydrate, which seems to be incompletely oxidized (negative fat values); but that sometime during neurulation a maximum of the rate of carbohydrate consumption is reached, and the utilization of fat starts.

With the increasing rate of fat combustion, the rate of carbohydrate oxidation approaches zero. As we shall discuss later, the calculated carbohydrate consumption agrees quite well with the difference in carbohydrate content between early and late stages found by direct chemical analysis. It is therefore reasonable at this stage to use equations **(5)** and **(6)**, which were based on simultaneous fat and protein combustion. Doing so, we find that together with the increasing rate of oxygen uptake, the rates of both fat and protein oxidation increase. In the interval

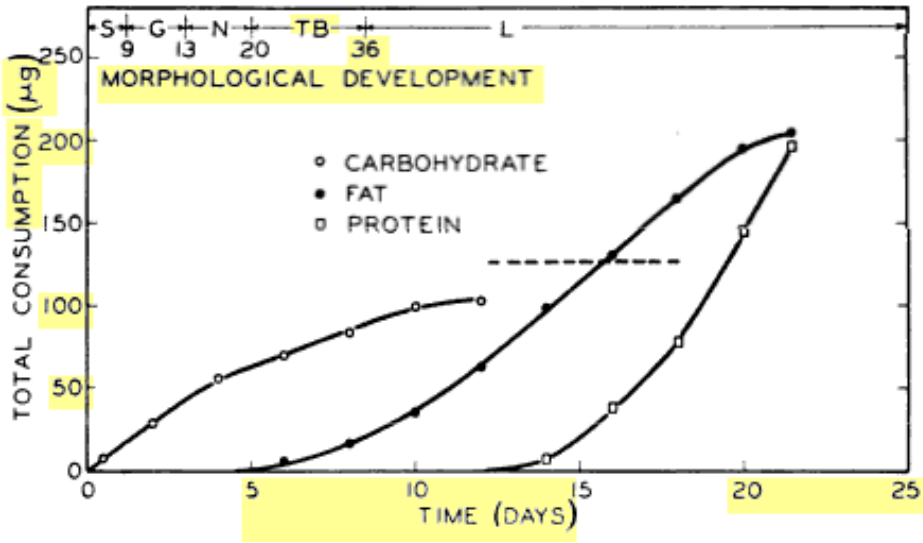

Fig. 6. Total consumption of energy sources. The stippled line represents the value of carbohydrate consumption expected from chemical analysis. For explanation of abbreviations, see figure 3.

between the 17th and 19th days the rate of fat oxidation begins to decrease, while the consumptions of protein and oxygen continue to increase until a maximum is reached after 22 days.

In fig. 6 the same values have been plotted, but using the total consumption rather than the rate of consumption as the ordinate. It is seen that the calculated total consumption of carbohydrate (102 μg) does not reach completely the value expected from the chemical determinations (126 μg; stippled line), but this can be explained as being due to incomplete oxidation. The value of negative fat consumption (9 μg) would correspond to about 9 μg carbohydrate not registered, and if this rate of incomplete carbohydrate oxidation continued also when we cannot detect it due to simultaneous fat oxidation, we would closely approach the level indicated in the figure. On the other hand it should be recalled that both

BRACHET and NEEDHAM (12) and GREGG (15) found constancy of TC during the segmentation stages, while the present calculations indicate a loss. The chemical analyses must of course be decisive on this point. Apparently the substance oxidized during this time causes a slight decrease in RW, which in the present analysis can only be registered as due to carbohydrate combustion. This phenomenon would have the opposite effect, so that all values are about 7 μg too high. Nevertheless, there is no doubt that we would get a closer approximation to the stippled line, if it were possible to correct the calculated curve. On the whole the main difference between this carbohydrate curve and those found by BRACHET and NEEDHAM and by GREGG seems to be that the depletion of carbohydrate occurs at a morphologically earlier stage in this case.

The fat consumption curve (negative values are not included) shows that fat combustion starts sometime about the end of neurulation or the beginning of the tailbud stages. The total amount consumed at the time of maximum respiration is 204 µg or 41 per cent of the total amount of lipoids (500 µg) and 58 per cent of the total amount of neutral fat (350 µg) calculated to be present at fertilization.

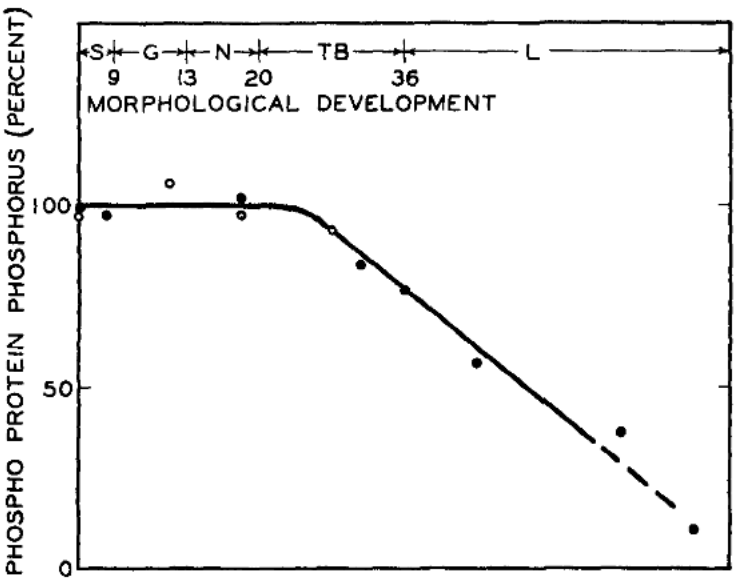

Fig. 7. The change in phosphoprotein phosphorus during development. For explanation of abbreviations, see figure 3. The open and filled circles represent two different batches. The time has not been used as abscissa, because embryos from different batches, having different rates of development, have been used. To enable plotting of these data in the same figure, the morphological development has been used as abscissa.

It should be recalled that these values are minimum values. If the oxidation of fat and protein has been incomplete, the true values will be correspondingly greater.

The calculations show that protein is being used as an energy source, starting some time during the larval development. The amount used up to the respiratory maximum was 195 μg, or 16 per cent of the total amount present in the eggs.

5.4. The changes in phosphoprotein phosphorus (PPP).

The results of the determinations of *PPP* are shown in fig. 7. The absolute values (corresponding to 100 per cent) were for the two batches 19.4 and 15.9 μg *PPP* per embryo. The curve indicates that the utilization starts some time during the tailbud stages. The results of BARTH and BARTH (3) showed that the utilization starts only during larval development, while KUTSKY'S results (24) seem to indicate that the utilization starts even earlier than found here. The number and the accuracy of the present determinations do not permit a decision between these alternatives.

6. DISCUSSION

6.1. Energy sources and yolk utilization.

A comparison with the results reviewed in section 2.1 will show that the present results agree with the majority of the previous results in showing that carbohydrate and fat are the main energy sources of the embryonic development in amphibia. Of the total amount of carbohydrate present at fertilization, 60-70 per cent (*i.e.*, all the glycogen) is consumed, the oxidation, in the present case at least, being incomplete. Of the total amount of neutral fat at least 58 per cent (corresponding to 41 per cent of the lipoids present) has been consumed at the time of the respiratory maximum. At the same time about 16 per cent of the total protein has been combusted.

These results also confirm the utilization of protein as an energy source, but it is seen (fig. 5) that this happens only at a very late stage of larval development. WILLS' results (41) indicate that this combustion of protein may be avoided, or at least kept at a low level, if food uptake is allowed. The loss of *TN* found in the present experiments (16 per cent) corresponds approximately to the highest value found by WILLS. The still higher values found by BARTHÉLEMY and BONNET (2) and by BIAŁASZEWICZ and MINCOVNA (7) (25 and 50 per cent, respectively) probably indicate that the animals were

more exhausted than at the end of the present experiments, although the value of 50 per cent seems to be exceptionally high (cf. the following paper (28)).

In view of the evidence presented it seems necessary to modify NEEDHAM'S generalization (32; 33) with respect to energy sources, as far as amphibian embryos are concerned. The correct succession in this case is: Carbohydrate, fat, and, provisionally, protein. The results obtained (see fig. 5) seem furthermore to indicate that a new kind of material is mobilized for energy supply when the rate of supply of the preceding one can no longer be increased to meet the demands (cf. the discussion in the following paper (28)).

It was mentioned in section 2.2 that all carbohydrate, 30–50 per cent of the lipoid material (fatty acids), and 30 per cent of *TN* were found outside the yolk. A comparison of these values with the values of consumption shows that quantitatively there is no basis for concluding anything about yolk utilization from the combustion going on. It must be emphasized that this statement is concerned only with the quantitative aspect of the question, it does not of course imply that the molecules originally found in the yolk are used exclusively for synthetic purposes, and none oxidized. The question of yolk utilization seems better answered by analysis of the yolk nitrogen (= non-extractable nitrogen) as done by GREGG and BALLENTINE (16), or by analysis of phosphoprotein phosphorus, as done by KUTSKY (24) and by BARTH and BARTH (3) and in the present work. It should be emphasized, however, that these two approaches need not give the same answer, since yolk phosphorus might very well be utilized for synthetic purposes, without the simultaneous use of yolk nitrogen. The results of GREGG and BALLENTINE (16) and BARTH and BARTH (3) seem, however, to be in good agreement in showing that yolk utilization does not start until the beginning of larval development. The present experiments indicate the onset of yolk utilization to occur slightly earlier during the morphological development. Protein combustion starts at a much later stage of development , and it is thus seen that yolk utilization, as reflected by the decrease in *PPP*, is not associated with any protein combustion, but presumably with a very active protein metabolism. It seems certain that little or no inactive reserve (yolk) protein is left at the time when protein combustion starts, *i.e.* the proteins consumed have already been incorporated in the protoplasm (cf. the two succeeding papers (28; 29)).

As mentioned in section 2.2, BARTH and BARTH (3; 4) have found in their very interesting work on yolk utilization that the phosphate in yolk is bound in energy-rich phosphate bonds, thus constituting a potential source of energy. It would seem to be of some interest to compare the amount of energy stored in these phosphate bonds with the amount of energy made available by oxidative metabolism. The energy in energy-rich phosphate bonds is about 12000 cal/mol, and in phosphate ester bonds about 2000 cal/mol. As simultaneous analysis of *PPP* and *RNAP* shows, almost the total decrease in PPP can be accounted for by the rise in ribonucleic acid phosphorus (30); the phosphate must then be present as ester bonds after the transfer from yolk, and a loss in energy of about 10000 cal/mol occurs. The analysis shows that about 18 μg *P* is present in the egg as *PPP*. The loss in energy is accordingly 18/32 X 10^{-6} x 10000 cal = 0.56 x 10^{-2} cal. The oxidation of 102 μg carbohydrate, 204 μg fat (negative values excluded), and 195 μg protein, corresponding to a total oxygen consumption of about 662 μl O_2, (0°; 760 mm) is associated with a loss in energy of about 3.4 cal. The energy in the phosphate bonds thus corresponds to about 0.15 per cent of the total energy consumption during development. The energy quantities used in the calculations above are the potential quantities, nothing can be said about how much is utilized by the larvae.

6.2. The respiratory curves.

It was mentioned above that the curve for the oxygen uptake was horizontal during the period of neurulation and the first tailbud stages. The same phenomenon has been recorded in similar experiments carried out at other temperatures (15 °C, 20 °C, and 25 °C), although it was reflected only as a break at the two higher temperatures. At 15 °C, however, the same respiratory rate was found for four days (28). There is hardly any doubt that this phenomenon is the same as has been found by numerous investigators as a "break" in the respiratory curve. It might be argued that the two distinct curves (at 15 °C and 18 °C) found by the author are insufficient to decide the question of constancy. The same phenomenon has, however, been observed and described by TUFT, working with *Xenopus laevis* (39). In his experiments, TUFT has used an automatically recording respirometer (38), which of course is specially fit for deciding the question of constancy. In work where less refined techniques have been employed, there is some indication that the most distinct horizontal curves are obtained with urodele embryos, and at low

temperatures. Urodele embryos develop, at normal temperatures, more slowly than anuran embryos, and the rate is of course further retarded at low temperatures. The resulting very slow developmental rate makes it possible to make many determinations during the crucial phase, which means that the constancy may be established with greater certainty.

BOELL (8) has published a curve which, although it was not interpreted this way, indicates that a period of constant respiratory activity was observed in *Amblystoma mexicanum*. This period occurs somewhat later in the development than indicated by the results of TUFT and by the present experiments. The possible source of this discrepancy has been discussed by TUFT (39), who also convincingly disproves the frequent assertion that the changes in respiratory rate during amphibian embryogenesis follow a logarithmic curve –or rather two such ones with different logarithmic constants, joining at the "breaking" point. TUFT's arguments are supported by the results in this and the succeeding paper (28).

It has been suggested, to explain the shape of the respiratory curves, that the increase in respiratory rate follows the increase in cell number (ATLAS (1)), but this explanation seems too simplified. At least when one compares the curve showing the synthesis of *DNA* (measured as desoxyribosides) during the development (cf. HOFF-JØRGENSEN and ZEUTHEN (18)) with the respiratory curves, there are at least two facts which contradict ATLAS' suggestion. The first is that the relative increase in *DNA* (~1000 times (30)), and the increase in cell number must be considerably higher, as *DNA* (remains constant during segmentation) is much higher than the relative increase in respiratory rate (~ 20 times). The second fact is that the rate of oxygen consumption remains constant during the period in which the rate of *DNA* synthesis (and accordingly the increase in cell number) is highest (18), while the respiration increases continuously during the larval development although *DNA* synthesis almost stops for a certain period during this phase (18; 30).

It is impossible to correlate the shape of the respiratory curves with other observed phenomena. All we can do at present is to describe them as curves with several phases. The first phase is during segmentation, when the respiratory rate increases only slighty; during the next phase, comprising gastrulation and early neurulation, the rate increases considerably. During the rest of neurulation and the first tailbud stages, the rate of oxygen consumption remains constant. After this phase the respiratory rate increases continuously

until a maximum is reached. If food is provided, the increase continues after a period of constant or slightly reduced rate of respiration (WILLS (41)).

SUMMARY

The combustion of energy sources during the embryonic development of *Amblystoma mexicanum* has been followed by determinations of the changes in reduced weight and oxygen consumption. The interpretation and calculation of the data have been supported partly by total carbohydrate and total nitrogen analyses, and partly by similar analyses carried out by other investigators. It was found that the succession of energy sources is carbohydrate, fat, protein. Of the carbohydrate present in the egg 60-70 per cent, corresponding to about 125 μg, was utilized, partly without any simultaneous oxygen uptake. At the time of maximum respiration (22 days), which was chosen as endpoint in the calculations, 204 μg, *i.e.* 41 per cent of the total lipoids, or 58 per cent of the neutral fat, was combusted. At the same time 195 μg or 16 per cent of the protein was combusted, but it was concluded that this amount might have been lower if feeding had been allowed. Analysis of the changes in phosphoprotein phosphorus showed that the yolk phosphorus is not utilized before the tailbud stages, and that yolk utilization is not directly related to protein combustion.

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